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HIGHLIGHTS

Basic

FRONTLINE | Research Article

Specific microbiota enhances intestinal IgA levels by inducing TGF-β in T follicular helper cells of Peyer's patches in mice

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In humans and mice, mucosal immune responses are dominated by IgA antibodies and the cytokine TGF-β, suppressing unwanted immune reactions but also targeting Ig class switching to IgA. It had been suggested that eosinophils promote the generation and maintenance of mucosal IgA-expressing plasma cells. Here, we demonstrate that not eosinophils, but specific bacteria determine mucosal IgA production. Co-housing of eosinophil-deficient mice with mice having high intestinal IgA levels, as well as the intentional microbiota transfer induces TGF-β expression in intestinal T follicular helper cells, thereby promoting IgA class switching in Peyer's patches, enhancing IgA$^+$ plasma cell numbers in the small intestinal lamina propria and levels of mucosal IgA. We show that bacteria highly enriched for the genus *Anaeroplasma* are sufficient to induce these changes and enhance IgA levels when adoptively transferred. Thus, specific members of the intestinal microbiota and not the microbiota as such regulate gut homeostasis, by promoting the expression of immune-regulatory TGF-β and of mucosal IgA.

Keywords: *Anaeroplasma* · mucosal IgA · Peyer's patches · T follicular helper cells · TGF-β

See accompanying commentary by Eberl and Vieira

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Antibodies of the IgA isotype are dominant at mucosal surfaces in humans and mice. IgA antibodies are an integral part of the mucosal barrier, controlling the growth and attachment of specific bacteria [1]. Ig class switching to IgA occurs mainly in GC reactions of Peyer's patches (PP) [2], but can also occur in mesenteric lymph nodes, in isolated lymphoid follicles and in the small intestinal lamina propria (siLP), where B cells are activated without the help of T cells [3–5]. Class switch recombination is targeted to IgA by TGF-β [6, 7], which induces IgA switch transcripts [8,9], an essential prerequisite for IgA antibody class switch recombination [10]. In PP, the class switch to IgA is regulated by T follicular helper (Tfh) cells expressing TGF-β and IL-21 [11,12] and by dendritic cells activating latent TGF-β [13]. An important role in the promotion of mucosal IgA expression has been attributed to eosinophils [14,15]. It has been reported that ΔdblGATA-1 mice, which lack eosinophils [16], have reduced numbers of IgA^+ GC B cells in their PP, reduced numbers of IgA-expressing plasma cells in their siLP, and lower levels of mucosal IgA than WT mice [14, 15]. How eosinophils that are not present in PP can contribute to IgA class switching in PP had remained enigmatic. In the study of Chu and colleagues, ΔdblGATA-1 mice and WT control mice had been derived from different animal facilities, with different composition of the microbiota. Here, we report that allowing the exchange of microbiota by cohousing equalizes their levels of mucosal IgA.

It has been known for some time that the expression of mucosal IgA is also dependent on microbiota. Germ-free mice show reduced production of intestinal IgA, unless they are repopulated with bacteria [17,18]. So far, members of the intestinal microbiota that enhance IgA expression specifically and selectively have not yet been identified. Here, we report the serendipitous observation when comparing the microbiome of ΔdblGATA-1 mice and WT control mice that only the presence of bacteria of the genus *Anaeroplasma* in the microbiota correlated with increased TGF-β expression in Tfh cells of PP, enhanced class switch recombination to IgA in GC B cells of the PP, increased numbers of IgA^+ plasma cells in the siLP, and enhanced expression of mucosal IgA. Adoptive transfer of bacteria highly enriched in *Anaeroplasma* and depleted for most other bacteria into secondary abiotic mice resulted in the generic induction of high intestinal IgA levels, supporting the notion that *Anaeroplasma* specifically and not bacteria as such are dictating TGF-β and IgA expression in the small intestine. *Anaeroplasma* could thus have the potential as prime candidates for effective anti-inflammatory probiotics.

Results

The intestinal microbiota determine mucosal IgA levels independently of eosinophils

It had been previously described that ΔdblGATA-1 mice, lacking eosinophils, also have reduced mucosal IgA, suggesting that eosinophils are regulating mucosal IgA production [14,15]. In view of recent findings, that the phenotypic manifestation of a particular genotype can be dependent on the microbiota (reviewed in [19]), we cohoused ΔdblGATA-1 mice bred and maintained at the German Rheumatism Research Center (DRFZ) with BALB/c mice purchased from Charles River for 3 weeks to allow for the exchange of microbiota by coprophagy. Cohousing of ΔdblGATA-1 mice and BALB/c mice equalized their expression of mucosal IgA. Non-cohoused ΔdblGATA-1 mice expressed 0.035 ± 0.003 μg/mg of fecal IgA, and non-cohoused BALB/c mice about four times more (0.166 ± 0.020 μg/mg; Fig. 1A). The absolute number of IgA^+B220^- plasma cells ($1.28 \pm 0.34 \times 10^5$) was more than 10-fold lower in the siLP of non-cohoused ΔdblGATA-1 mice when compared to BALB/c mice ($15.11 \pm 2.30 \times 10^5$; Fig. 1B and C), and in the PP of non-cohoused ΔdblGATA-1 mice the numbers of $IgA^+PNA^{hi}B220^+$ GC B cells were more than 20-fold lower than in non-cohoused BALB/c mice ($6.50 \pm 1.29 \times 10^3$ vs. $145.1 \pm 30.3 \times 10^3$; Fig. 1D, E, and G). Interestingly, while the frequency of $IgG1^+$ GC B cells was increased in the PP of ΔdblGATA-1 (Fig. 1D), absolute numbers of switched $IgG1^+$ GC B cells did not differ between non-cohoused mice of the two strains ($5.05 \pm 1.75 \times 10^3$ vs. $3.43 \pm 0.96 \times 10^3$; Fig. 1F). Fecal IgA concentrations were not significantly different between cohoused ΔdblGATA-1 mice and BALB/c mice (0.163 ± 0.046 μg/mL vs. 0.145 ± 0.039 μg/mL; Fig. 1A). Absolute numbers of IgA^+ plasma cells in the siLP ($19.32 \pm 4 \times 10^5$) and of IgA^+ GC B cells in the PP of ΔdblGATA-1 mice ($60.92 \pm 21.65 \times 10^3$) were no longer significantly different from those of BALB/c mice ($21.96 \pm 3.79 \times 10^5$ and $189.4 \pm 64.8 \times 10^3$; Fig. 1B–E and G). Numbers of $IgG1^+$ GC B cells in the PP were not affected by cohousing (Fig. 1D and F). Taken together, these data show that eosinophils are dispensable for the regulation of mucosal IgA expression, and more importantly, that distinct components of the murine microbiota, rather than the microbiota as such, regulate mucosal IgA expression. In line with this notion, mice from any strain of the DRFZ breeding facility had consistently lower titers of fecal IgA (<0.05 μg IgA/mg feces) than mice of the strains we obtained from the Charles River breeding facility (>0.08 μg IgA/mg feces; Supporting Information Fig. 1).

The fecal microbiome composition of BALB/c and ΔdblGATA-1 mice under non-cohoused and cohoused conditions was compared by next-generation 16S rDNA sequencing of V3/V4 regions. The fecal microbiome was substantially different between ΔdblGATA-1 from the DRFZ and BALB/c mice from Charles River (Fig. 1H). However, we did not observe substantial differences in the number of bacterial genera detected, or in Shannon and Simpson diversity indices of the fecal microbiome of BALB/c and ΔdblGATA-1 mice (Supporting Information Fig. 2A–C). Following 3 weeks of cohousing, Bray-Curtis similarity clustering on the family level revealed that the microbiomes between BALB/c and ΔdblGATA-1 can no longer be distinguished (Fig. 1I and J), indicating an exchange of the microbiota by coprophagy.

The IgA^{high} phenotype is transferred in a dominant fashion by the microbiota

To confirm that (1) the IgA phenotype is conferred by the microbiota and (2) is independent of the ΔdblGATA-1 genotype, we

intentionally transferred, by gavage, the fecal microbiota from either IgA^{high} BALB/c (Charles River) or IgA^{low} ΔdblGATA-1 (DRFZ) mice into BALB/c mice, which had been treated for 2 weeks with a cocktail of antibiotics, containing ampicillin, metronidazole, vancomycin, and neomycin, ablating their own microbiota (Fig. 2A). Twenty-one days after gavage, mice that had received IgA^{high} microbiota had significantly higher levels of fecal IgA (0.231 ± 0.036 μg/mg feces) and serum IgA (82.05 ± 5.09 μg/mL), as compared to mice receiving the IgA^{low} microbiota (fecal IgA: 0.103 ± 0.034 μg/mg feces; serum IgA: 64.42 ± 5.67 μg/mL; Fig 2B and C). Absolute numbers of $CD45^+IgA^+B220^{lo}$ plasma cells in the siLP of IgA^{high} microbiota recipients were about twofold higher (26.83 ± 3.75 × 10^5) than those of IgA^{low} microbiota recipients (14.24 ± 2.47 × 10^5; Fig. 2D and E). The absolute numbers of $IgA^+PNA^{hi}B220^+$ GC B cells in the PP of IgA^{high} microbiota recipients were about threefold increased (4.13 ± 0.78 × 10^3), compared to IgA^{low} microbiota recipients (1.38 ± 0.42 × 10^3; Fig. 2F and G). In contrast, the absolute numbers of $IgG1^+$ GC B cells in the PP were not significantly different between the two groups (2.03 ± 0.35 × 10^4 vs. 2.64 ± 0.69 × 10^4; Fig. 2F and H). The successful transfer of IgA-inducing microbiota of IgA^{high} BALB/c mice into microbiota-ablated BALB/c mice demonstrates that (1) BALB/c microbiota contains bacterial species inducing IgA in a dominant fashion, and (2) reduced microbiota-induced IgA expression is not a peculiarity of ΔdblGATA-1 mice.

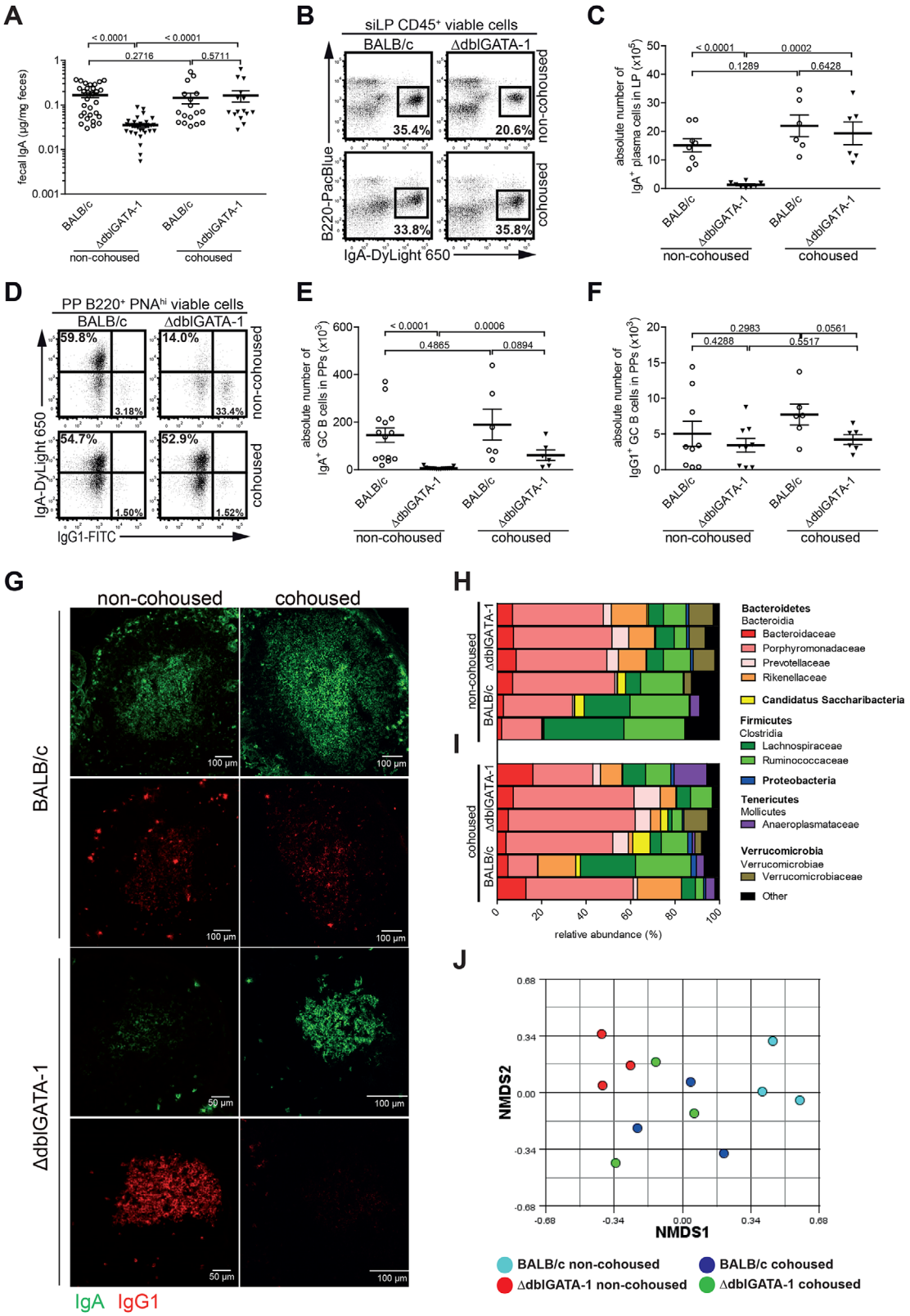
Specific members of the microbiota enhance mucosal IgA

To elucidate which bacterial species participate in inducing the IgA^{high} phenotype, we compared the microbiome of BALB/c and ΔdblGATA-1 mice filtering for bacterial OTUs that are present in BALB/c and non-detectable in ΔdblGATA-1 (Fig. 3A; Supporting Information Fig. 3) and that can be detected in ΔdblGATA-1 mice after cohousing with BALB/c mice (Fig. 3B). Of ≈100 bacterial OTUs detected in total (Supporting Information Fig. 2A), 19 OTUs were exclusively detected in the microbiome of BALB/c mice (Fig. 3A) and only two, *Anaeroplasma* and *Erysipelotrichaceae incertae sedis*, were consistently detected in ΔdblGATA-1 mice following cohousing (Fig. 3B), thus qualifying them as prime candidates for the induction of IgA.

To analyze whether *Anaeroplasma* (Phylum *Tenericutes*) and/or *Erysipelotrichaceae incertae sedis* (Phylum *Firmicutes*) can induce IgA, we separated them using a 0.45-μm pore size filter. *Tenericutes* are 0.2–0.5 μm in size [20], smaller than most bacteria of the intestinal microbiota. *Anaeroplasma* were the only representatives of the phylum *Tenericutes* within the BALB/c intestinal microbiomes. By filtration of the fecal microbiota of BALB/c mice, *Tenericutes,* i.e., *Anaeroplasma,* could be separated from *Erysipelotrichaceae incertae sedis* and from all other genera, which were identified before to be specific for the microbiome of our BALB/c mice (Fig. 3C). *Tenericutes* could be enriched by several orders of magnitude by filtration of the fecal microbiota of BALB/c mice (Fig. 3D). The *Anaeroplasma*-enriched filtrate was then transferred into antibiotics-treated, microbiota-ablated BALB/c mice (Fig. 3E). The filtrate was supplemented with *Anaeroplasma*-negative ΔdblGATA-1 microbiota, since it has been suggested that some *Anaeroplasma* strains are auxotrophs lacking sterol synthesizing capability [20]. Transfer of *Anaeroplasma*-enriched filtrate enhanced expression of fecal IgA from 0.255 ± 0.017 to 0.469 ± 0.055 μg/mg, comparable to the transfer of the entire BALB/c microbiota (from 0.307 ± 0.014 μg/mg feces to 0.612 ± 0.051 μg/mg feces), strongly suggesting that *Anaeroplasma* are indeed IgA inducing bacteria (Fig. 3F). Mice not having received any microbiota after antibiotic treatment showed no increase in their fecal IgA concentration (Supporting Information Fig. 4A). The serum IgA concentration was not affected by the presence of *Anaeroplasma* in the transferred microbiota (Supporting Information Fig. 4B). Numbers of IgA^+ plasma cells in the siLP were significantly increased in recipients of *Anaeroplasma*-enriched microbiota in comparison to recipients of microbiota lacking *Anaeroplasma* (Fig. 3G and H). Since filtration of BALB/c microbiota through a 0.1-μm filter did not augment IgA levels (Supporting Information Fig. 4C), the presence of the bacteria, rather than any secreted product, is the main drivers of IgA induction. By FISH using an *Anaeroplasma*-specific 16S rDNA probe, we could localize *Anaeroplasma* in the small intestine of BALB/c mice (Fig. 3I). *Anaeroplasma* could not be detected by FISH in the large intestine of BALB/c mice or the small intestine of ΔdblGATA-1 mice (Fig. 3J).

Generic induction of IgA by *Anaeroplasma*-containing microbiota

To determine whether the presence of *Anaeroplasma* results in the general, generic enhancement of IgA levels or rather induces IgA of distinct specificities, e.g., against *Anaeroplasma* itself, we stained fecal bacteria of $Rag1^{-/-}$ mice, lacking own IgA, with fecal IgA of BALB/c mice having received *Anaeroplasma* (−) or (+) microbiota (Supporting Information Fig. 5A). Staining of the IgA-coated bacteria was then evaluated by high-resolution microbiota flow cytometry [21]. Microbiota stained with IgA of *Anaeroplasma* (−) and (+) mice showed the same cytometric profile (Supporting Information Fig. 5B and C), and sequencing of IgA-bound bacteria (IgA-Seq [22]) confirmed that the composition of IgA-bound bacteria was indistinguishable between the two groups (Supporting Information Fig. 5E–G). However, the $Rag1^{-/-}$ microbiota were stained two to four times brighter (MFI) by IgA of *Anaeroplasma* (+) mice (Supporting Information Fig. 5D), corresponding to the difference in fecal IgA levels observed between *Anaeroplasma* (+) and (−) mice (Fig. 3F). Thus, *Anaeroplasma* appear to enhance mucosal IgA responses, irrespective of the antigen targeted, and qualify as an IgA enhancing adjuvant. It may be also effective in humans, since fecal IgA levels of healthy human donors correlate to the amount of *Anaeroplasma* detected by quantitative PCR in these feces (Supporting Information Fig. 6).



Anaeroplasma induce TGF-β expression in T follicular helper cells of Peyer's patches

The selective and dominant effect of *Anaeroplasma*-enriched microbiota on absolute numbers of $IgA^+PNA^{hi}B220^+$ GC B lymphocytes in the PP (Figs. 1E and 2G) suggests that *Anaeroplasma* directly influence the regulation of antibody class switch in B lymphocytes. TGF-β is the cytokine targeting antibody class switch recombination to IgA [6–9], and in PP, it is expressed by Tfh controlling activation and differentiation of B lymphocytes [12]. We isolated $CD3^+CD4^+CXCR5^+PD\text{-}1^+$ Tfh and $CD3^+CD4^+CXCR5^-PD\text{-}1^-$ non-Tfh cells from PP of BALB/c mice, ΔdblGATA-1 mice, and of ΔdblGATA-1 mice cohoused with BALB/c mice for 3 weeks (Supporting Information Fig. 7A). Expression of *Tgfb1* mRNA was determined in these cells by quantitative RT-PCR directly ex vivo. Tfh cells of *Anaeroplasma*-deficient ΔdblGATA-1 mice express significantly less *Tgfb1* than Tfh cells of *Anaeroplasma*-sufficient BALB/c mice and those of co-housed ΔdblGATA-1 mice (Fig. 4A). This effect was selective for TGF-β, since *Il4* mRNA expression did not differ between Tfh of the various mice (Fig. 4B). It was also selective for Tfh lymphocytes, since in MHC class II-positive cells of the siLP and in non-Tfh of PP *Tgfb1* expression did not differ between *Anaeroplasma*-sufficient and -deficient mice (Supporting Information Fig. 7B and C). In intestinal epithelial cells, *Tgfb1* transcript was not detectable (data not shown). In vitro, a 0.45-μm filtrate of BALB/c microbiota containing *Anaeroplasma* could significantly enhance *Tgfb1* expression by a factor of 3 in splenocytes activated with anti-CD3 and anti-CD28 antibodies, as compared a 0.45-μm filtrate of ΔdblGATA-1 microbiota, not containing *Anaeroplasma* (Fig. 4C), and control cultures with no filtrate added. Apparently, the induction of TGF-β expression in Tfh of PP is the mechanism by which *Anaeroplasma* enhance mucosal IgA expression.

Discussion

IgA antibodies [23] and the cytokine TGF-β [24] dominate mucosal immunity. It is less clear how TGF-β and IgA are targeted in mucosal immune reactions. Here, we report identification of a distinct member of the intestinal microbiota, which generically enhances mucosal IgA expression in a dominant fashion, probably by inducing in mucosal Tfh the expression of TGF-β, the cytokine targeting antibody class switch recombination to IgA [8,9]. So far, the species-specific induction of IgA immune reactions by bacteria of the microbiota has been described [17,18,22], but it has been unclear whether such bacteria induce mucosal immune reactions predisposed to TGF-β/IgA, or whether they themselves target TGF-β and/or IgA expression.

Eosinophil-deficient ΔdblGATA-1 mice had been described to be impaired for mucosal IgA [14,15], however, the role of differences in the microbiota between ΔdblGATA-1 and their WT BALB/c counterpart were not considered in these studies. We cohoused eosinophil-deficient ΔdblGATA-1 bred and maintained at the animal facility of the DRFZ with BALB/c mice obtained from Charles River for 3 weeks and could show, that ΔdblGATA-1 mice increase expression of mucosal IgA, numbers of IgA^+ GC B cells in their PP, and numbers of IgA^+ plasma cells in their siLP to the same level as BALB/c mice. The assumption that the enhancement of mucosal IgA expression in ΔdblGATA-1 mice had been due to an uptake of BALB/c microbiota by coprophagy was confirmed by oral gavage of BALB/c microbiota into antibiotics-treated, secondary abiotic BALB/c mice. In these mice, only the microbiota of BALB/c mice but not that of ΔdblGATA-1 mice reconstituted mucosal IgA levels. Below the line, these data show that the microbiota of BALB/c mice from the Charles River animal facility contains bacteria that are able to enhance mucosal IgA expression.

By comparing the microbiomes of the ΔdblGATA-1 with the BALB/c mice using 16S rDNA sequencing, we could identify only two bacterial genera correlated to the enhanced expression of IgA and were transferred by cohousing from BALB/c mice into ΔdblGATA-1 mice, namely *Anaeroplasma,* and *Erysipelotrichaceae incertae sedis. Anaeroplasma* and *Erysipelotrichaceae incertae sedis* could be physically separated by filtration, and only *Anaeroplasma* enhanced mucosal IgA expression in antibiotics-treated, secondary abiotic BALB/c mice. Presumably, *Anaeroplasma* do so in a unique way, namely by enhancing expression of TGF-β in Tfh of the PP. TGF-β-receptor signaling in B cells targets antibody class switch recombination to IgA [6,25,26] inducing Iα germline transcripts [27–29]. Several cell types of the mucosa have been shown to be able to produce TGF-β: Dendritic cells of PP [30–32], intestinal epithelial cells (IEC) [33], and PP follicular dendritic cells [34]. These cells express TGF-β upon stimulation of TLR signaling. This

Figure 1. The microbiota regulates expression of mucosal IgA in eosinophil-deficient ΔdblGATA-1 mice. IgA^{high} BALB/c and IgA^{low} ΔdblGATA-1 mice were cohoused for 3 weeks. (A) IgA levels in feces of IgA^{high} BALB/c and IgA^{low} ΔdblGATA-1 mice under non-cohousing and cohousing conditions as measured by ELISA. (B and C) Flow cytometric determination of viable $CD45^+B220^{lo}IgA^+$ siLP plasma cells in non-cohoused and cohoused IgA^{high} BALB/c and IgA^{low} ΔdblGATA-1 mice. Exemplary flow cytometry plots (B), respectively flow cytometric determination of absolute numbers of $CD45^+B220^{lo}IgA^+$ siLP plasma cells (C) are shown. (D–F) Flow cytometric determination of viable $B220^+PNA^{hi}IgA^+$ and $B220^+PNA^{hi}IgG1^+$ PP GC B cells in non-cohoused and cohoused IgA^{high} BALB/c and IgA^{low} ΔdblGATA-1 mice. Exemplary flow cytometry plots (D), respectively flow cytometric determination of absolute numbers of $B220^+PNA^{hi}IgA^+$ (E) and $B220^+PNA^{hi}IgG1^+$ (F) PP GC B cells in non-cohoused and cohoused IgA^{high} BALB/c and IgA^{low} ΔdblGATA-1 mice are shown. (G) Immunofluorescence staining of IgA (green) and IgG1 (red) in PP of BALB/c and ΔdblGATA-1 mice under non-cohoused and cohoused conditions. One representative image of $n = 3$ mice per group from single experiment is shown. Scale bar: 50 or 100 μm, 20× magnification (H and I) Relative abundance of main bacterial families as determined by 16S rDNA deep sequencing for non-cohoused (H) and cohoused (I) BALB/c and ΔdblGATA-1 mice for $n = 3$. (J) Non-metric multidimensional scaling (NMDS) on family level using the Bray-Curtis distance for non-cohoused and cohoused BALB/c and ΔdblGATA-1 mice for $n = 3$. Error bars indicate mean ± SEM for (A) n = 15–30 mice from five independent experiments and (B–F) $n = 6$–8 mice from two independent experiments. p-Values determined by an unpaired two-tailed Student's t-test. (H–J) Shown are the data for $n = 3$ from single representative experiment.

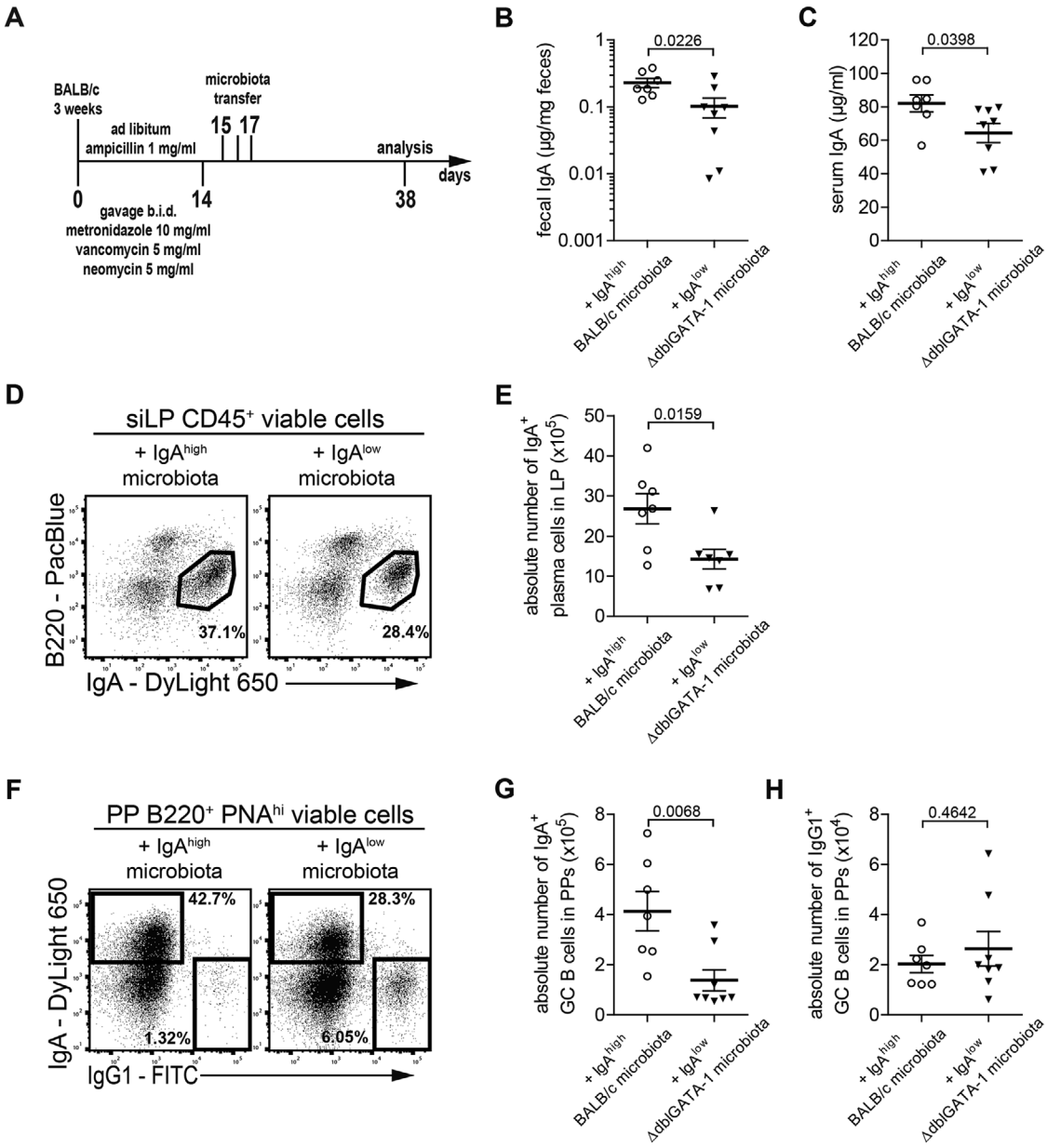


Figure 2. Induction of mucosal IgA is dependent on the composition of the microbiota. (A) Six-week-old BALB/c mice treated for 14 days with an antibiotics cocktail (metronidazole 10 mg/mL, vancomycin 5 mg/mL, neomycin 5 mg/mL per gavage (200 µL) b.i.d., and ampicillin 1 mg/mL ad libitum via the drinking water) were transferred with fecal microbiota from BALB/c or ΔdblGATA-1 mice on three consecutive days by gavage. Three weeks after microbiota transfer the mice were analyzed. (B and C) IgA levels in feces (B) and blood sera (C) as measured by ELISA. (D and E) Flow cytometric determination of viable $CD45^+B220^{lo}IgA^+$ siLP plasma cells. Exemplary flow cytometry plots (D), respectively flow cytometric determination of absolute numbers of $CD45^+B220^{lo}IgA^+$ siLP plasma cells (E) are shown. (F and H) Flow cytometric determination of viable $B220^+PNA^{hi}IgA^+$ and $B220^+PNA^{hi}IgG1^+$ PP GC B cells. Exemplary flow cytometry plots (F), respectively flow cytometric determination of absolute numbers of $B220^+PNA^{hi}IgA^+$ (G) and $B220^+PNA^{hi}IgG1^+$ (H) PP GC B cells are shown. Error bars indicate mean ± SEM for $n = 8$ mice from two independent experiments (B–H). *p*-Values determined by unpaired two-tailed Student's *t*-test.

may be a mechanism to sense microbiota as such and provide a basic level of expression of TGF-β and IgA. It should be noted that *Anaeroplasma*, like all members of the constituting phylum *Tenericutes*, do not have a cell wall [20] and could potentially escape notice of inflammatory cell wall-detecting pattern recognition receptors. Obviously, *Anaeroplasma*, enhancing this basic IgA expression two- to fivefold, is not using this pathway, since we did not observe any difference in TGF-β expression in MHC class II^+ cells of PP or non-Tfh cells of PP and no expression of TGF-β in IEC of *Anaeroplasma*-deficient and -sufficient mice. However, Tfh of PP differed significantly in their transcription of the *Tgfb1* gene. Tfh cells regulate T-dependent (TD) mucosal IgA responses [12,35]. In vitro, *Anaeroplasma* also induce *Tgfb1* transcription in activated splenocytes. So far, the mechanism is unclear, but it is

obviously mediated by the bacterium itself and not a secreted product, since the 0.1 μm filtrate of *Anaeroplasma*-containing microbiota does not enhance mucosal IgA expression. By FISH, we localized Anaeroplasma to the small intestine of BALB/c but not ΔdblGATA1 mice, closely associated with the epithelium but not in PP, suggesting that in vivo *Anaeroplasma* induce *Tgfb1* expression in Tfh cells indirectly. In summary, *Anaeroplasma* qualify as a key anti-inflammatory component of the microbiota, since both, TGF-β, and IgA have been shown to protect from intestinal inflammation [36,37].

Bacteria that affect IgA levels in the gut have been described previously. Mice monocolonized with "segmented filamentous bacteria" (SFB) show increased IgA levels compared to mice colonized with *Escherichia coli*. This has been attributed to the vigorous induction of germinal centers in secondary and tertiary lymphoid structures in the intestine [38]. In the BALB/c and ΔdblGATA-1 mice analyzed here, SFBs were present in both microbiomes. Bacterial metabolites, in particular short-chain fatty acids, have been shown to promote IgA responses in the gut. They apparently do so by boosting the differentiation of B cells into antibody-secreting plasma cells. The levels of systemic IgG increase, too [39]. Moon et al. have described a *Sutterella*-containing murine microbiota, which degraded secretory IgA [40]. The *Sutterella*-microbiota induced IgA^{low} phenotype is dominant. In contrast, *Anaeroplasma*-containing microbiota induces a dominant IgA^{high} phenotype. Obviously, *Anaeroplasma* are special among IgA regulating bacteria of the intestinal microbiota, in that they enhance TGF-β and IgA expression in TD mucosal immune reactions generically and impressively. We could show that also in humans, levels of fecal IgA correlate to levels of fecal *Anaeroplasma*. *Anaeroplasma* may thus qualify as a robust adjuvant or probiotic to enhance mucosal immunity. Although the cultivation of *Anaeroplasma* has been reported previously [41], we have not been able to reproduce these findings so far. Nevertheless, our data based on the isolation of Anaeroplasma by physical means provide compelling evidence that *Anaeroplasma* are an IgA and TGF-β inducing component of the intestinal microbiota.

Apart from the identification of *Anaeroplasma* as IgA and TGF-β inducing bacteria, the present experiments also show that eosinophils are dispensable for the regulation of mucosal IgA. In the presence of *Anaeroplasma*, ΔdblGATA-1 mice, deficient for eosinophils, show the same numbers of IgA^+ GC B cells in PP, IgA^+ plasma cells in siLP, and fecal IgA, as WT BALB/c mice.

Materials and methods

Mice

BALB/c and $Rag1^{-/-}$ mice were purchased from Charles River Laboratories, Sulzfeld, Germany. ΔdblGATA-1 [16] mice were bred under specific pathogen-free conditions in the DRFZ breeding facility at the Bundesinstitut für Risikobewertung, Berlin. All animal experiments were approved by the regulatory office "Landesamt für Gesundheit und Soziales" in Berlin, Germany (permit number G0045/17).

Cohousing and microbiota transplantation

For cohousing experiments, BALB/c and ΔdblGATA-1 females were kept in the same cage at a ratio of 1:1 for 3 weeks. For the depletion of the native intestinal microbiota, BALB/c mice were treated with ampicillin (1 mg/mL ad libitum) via the drinking water and with additional gavage feedings b.i.d. of neomycin 5 mg/mL, metronidazole 10 mg/mL and vancomycin 5 mg/mL (all purchased from Sigma, Darmstadt) for 14 days. Twenty-four hours after the last antibiotics administration the corresponding fecal microbiota was transferred via gavage on three consecutive days. For this, fresh fecal pellets of BALB/c (IgA^{high}) or ΔdblGATA-1 (IgA^{low}) mice were collected and resuspended in PBS (one pellet in 1 mL). Following filtration through a 30 μm cell strainer (Partec, Görlitz), 200 μL of the suspension were transferred via gavage feeding. The mice were analyzed 3 weeks after the last microbiota administration.

Determination of soluble IgA levels

Fresh feces were collected, weighed, and resuspended in PBS (1 mg feces in 10 μL PBS). The levels of fecal IgA in supernatants as well as in blood sera were determined by ELISA (ELISA antibodies purchased from Southern Biotech via BIOZOL Diagnostica, Eching).

Immunofluorescence of PP tissue sections

PP of non-cohoused and cohoused BALB/c and ΔdblGATA-1 mice were excised from the intestines, washed in ice-cold PBS, embedded in O.C.T. compound (Sakura Finetek; Alpen aan den Rijn, NL), and frozen. The frozen tissue was cut into 7 μm sections, which were fixed in 100% acetone (Carl Roth; Karlsruhe) for 20 min. For the immunofluorescence staining, the sections were blocked with anti-FcγR antibody (clone 2.4G2; DRFZ), following by the staining with the goat anti-mouse IgA-FITC (Southern Biotech via BIOZOL Diagnostica, Eching) and anti-mouse IgG1-PE (RMG1-1; Biolegend; Fell). The images acquired using a laser scanning microscope LSM 710 (Carl Zeiss) and ZEN2011 software.

Cell isolation

For the isolation of siLP cells, PP were removed and small intestines were longitudinally opened and washed in cold PBS. After cutting into short pieces, intestines were incubated twice in RPMI 1640 medium containing 25 mM EDTA, 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin (P/S) for 15 min

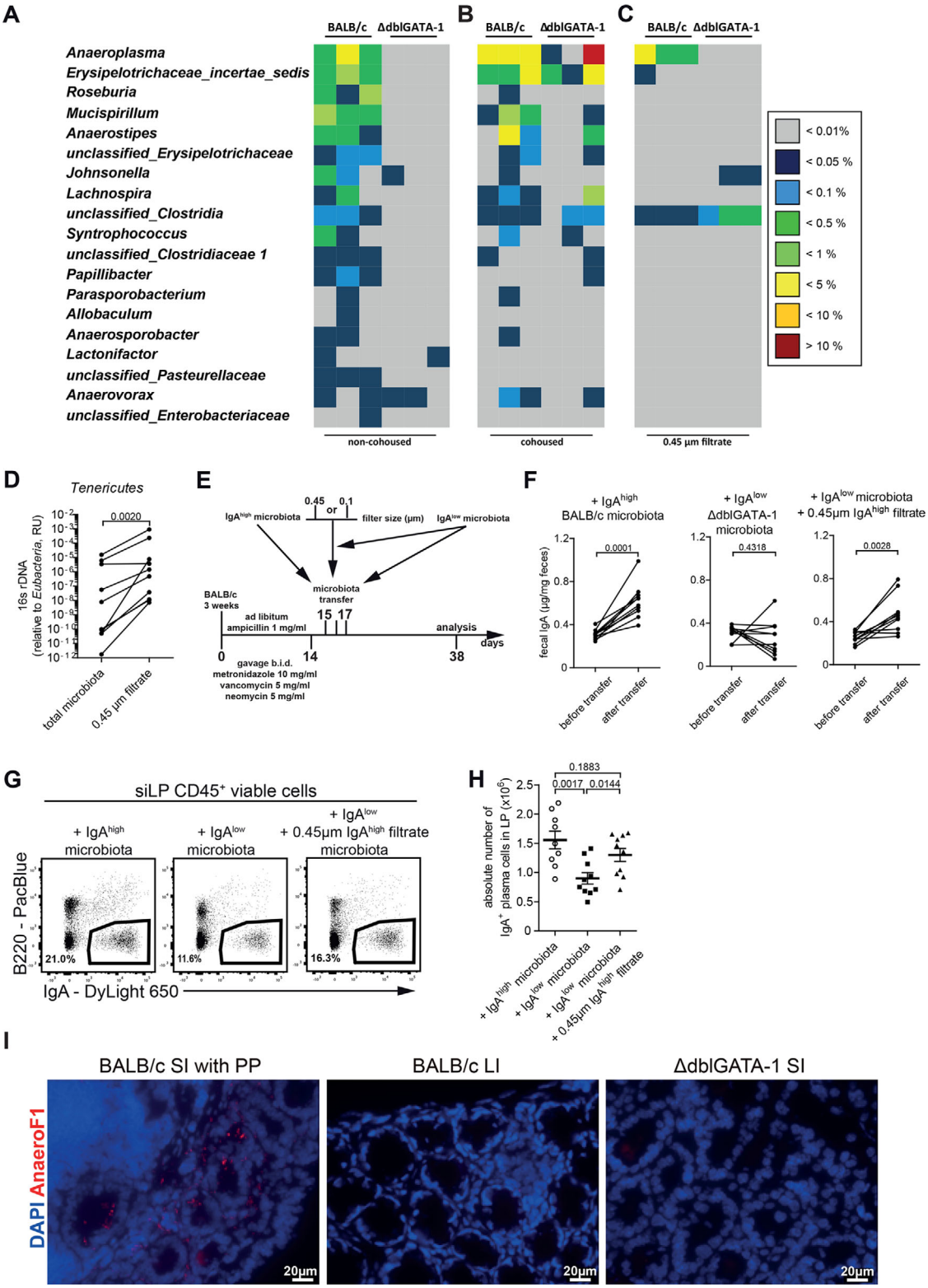


Figure 3. Defining of bacterial OTUs as candidates for IgA induction. (A–C) Abundance of differentially present bacterial OTUs in the microbiota of non-cohoused BALB/c mice and ΔdblGATA-1 mice (A) and their distribution following cohousing (B) based on 16S rDNA deep sequencing. (C) Abundance of the 19 OTUs in the 0.45-μm filtrate of BALB/c mice and ΔdblGATA-1 mice. (A–C) Shown are the data for $n = 3$ from single representative experiment. (D) Relative abundance of *Tenericutes* in the total fecal microbiota and in the 0.45-μm filtrate of fecal microbiota of BALB/c mice relative to total *Eubacteria* was measured by qRT-PCR. Lines connect the corresponding samples. Shown are the data for $n = 8$ from one representative experiment out of two independent experiments. *p*-Value determined by Wilcoxon matched-pairs signed rank test. (E) Three-week old BALB/c mice treated with antibiotics as described in Fig. 2A were transferred with IgA^{high} BALB/c, IgA^{low} ΔdblGATA-1, or IgA^{low} ΔdblGATA-1 fecal microbiota supplemented with the 0.45 μm filtrate of IgA^{high} BALB/c microbiota on three consecutive days by gavage. (F) IgA levels in feces of microbiota recipients directly after antibiotics treatment and 3 weeks after microbiota transfer as determined by ELISA. $n = 10$ mice from two independent experiments. (G and H) Flow cytometric determination of viable $CD45^+B220^{lo}IgA^+$ siLP plasma cells. Exemplary flow cytometry plots (G), respectively flow cytometric determination of absolute numbers of $CD45^+B220^{lo}IgA^+$ siLP plasma cells (H) are shown. Error bars indicate mean ± SEM for $n = 9$–10 mice from two independent experiments. *p*-Values determined by unpaired two-tailed Student's *t*-test. (I) Localization of *Anaeroplasma* by FISH in the small intestines of BALB/c (left), ΔdblGATA-1 (right) mice, and large intestine of BALB/c mice (middle). The tissue was counterstained with DAPI (blue). Scale bar: 20 μm, 60× magnification. Shown are representative images of three FISH-experiments with 3× BALB/c SI, 2× ΔdblGATA-1 SI, and 1× BALB/C LI organs.

at 37°C followed by vigorous shaking to remove the epithelium. Intestinal pieces were washed in PBS, homogenized using scalpel, and digested twice with RPMI 1640 containing 10% FCS, P/S, 1 mg/mL Collagenase D (Roche, Mannheim), 0,1 mg/mL DNase I, and 1 mg/mL Dispase II (Sigma, Darmstadt) for 20 min at 37°C in a shaker at 200 rpm. Cell suspensions were passed through an 18 gauge needle and filtered through a 70 μm cell strainer (BD Biosciences, Heidelberg). After washing with RPMI1640 medium (10% FCS, P/S), cells were purified by 30% Percoll (GE Healthcare, Hamburg) solution in RPMI 1640 medium (10% FCS, P/S). Cells were then washed and resuspended in RPMI 1640 medium (10% FCS, P/S). To isolate cells from PP, tissue was removed from the small intestine, meshed through a 70 μm cell strainer (BD Biosciences, Heidelberg) and resuspended in RPMI 1640, 10% FCS, P/S medium.

Flow cytometry

Flow cytometric analysis was performed in accordance with previously published guidelines .[42] Surface and intracellular staining was performed using following antibodies: CXCR5-Bio (L138D7), PD-1-APC (29F.1A12, BioLegend, Fell), B220-PacBlue (RA3.6B2), CD3-Bio (145-2C11), CD4-PB (GK1.5), all purified and conjugated at DRFZ, CD45-FITC (30-F11, eBioscience, Frankfurt, PNA-Bio (Vector Laboratories), and goat anti-mouse IgA-DyLight 650 (BIOZOL Diagnostica, Eching and BIOMOL GmbH, Hamburg). Intracellular IgG1-FITC (RMG1-1, BioLegend) staining was performed using the FoxP3 staining kit (eBioscience). Stained cells were acquired with a BD Canto II and data were analyzed with FlowJo V10 software. Tfh cells were sorted by flow cytometry with a BD Aria cell sorter as $CD3^+CD4^+CXCR5^{hi}PD\text{-}1^{hi}$ cells.

Gene-expression analysis

Total RNA was extracted using QIAzol Lysis Reagent (Qiagen, Hilden). The reverse transcription into the cDNA was performed with the Sensiscript RT kit (Qiagen). The expression of *Tgfb1* and *Il4* relative to *Actb* was measured by real-time PCR (95°C 30 s; 60°C 30 s, 72°C 30 s; 45 cycles) with the StepOnePlus (Applied Biosystems) using Maxima SYBR Green/ROX qPCR Master Mix

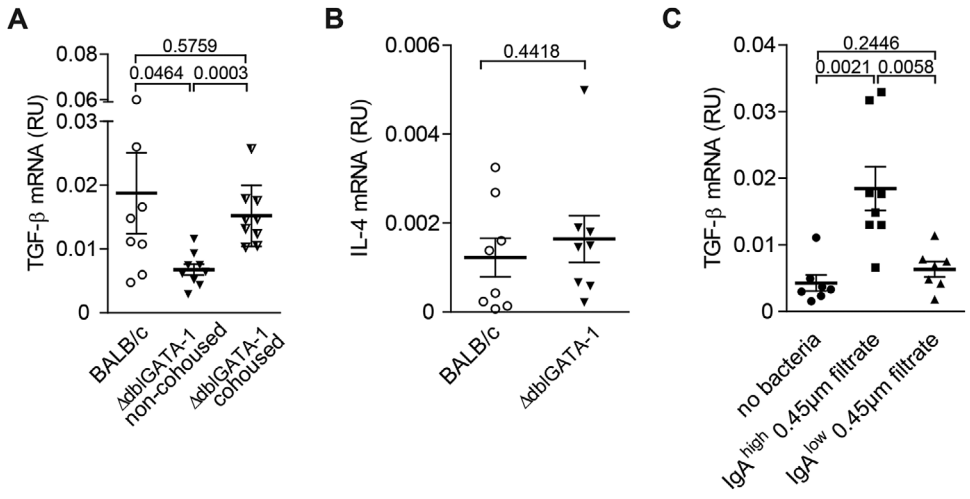


Figure 4. *Anaeroplasma* induce TGF-β expression in T follicular helper cells in Peyer's patches. (A) *Tgfb1* and (B) *Il4* mRNA expression relative to *Actb* mRNA analyzed by qRT-PCR in ex vivo isolated $CD3^+CD4^+CXCR5^+PD\text{-}1^+$ PP Tfh cells from BALB/c and non-cohoused and cohoused ΔdblGATA-1 mice. Error bars indicate SEM for $n = 8$–10 mice from three independent experiments. (C) *Tgfb1* mRNA levels relative to *Actb* mRNA in total splenocytes of BALB/c mice activated via CD3/CD28 in presence of 0.45 μm filtrates from IgA^{high} BALB/c or IgA^{low} ΔdblGATA-1 mice. Error bars indicate mean ± SEM for $n = 7$–8 from two independent experiments. *p*-Values determined by unpaired two-tailed Student's *t*-test.

from Thermo Scientific and the following primers: *Tgfb1* (Fw: 5′-CACAGCTCACGGCACCGGAGA-3′; Rv: 5′-GCTGTACTGTGTGTCCAGGCTCC-3′); *Actb* (Fw: 5′-CTCCTGAGCGCAAGTACTCTGTG-3′; Rv: 5′-TAAAACGCAGCTCAGTAACAGTCC-3′).

Analysis of fecal bacterial DNA

Bacterial DNA was isolated from frozen murine fecal pellets using ZR Fecal DNA MiniPrep Kit (Zymo Research, Freiburg) according to the manufacturer's protocol. The relative amount of specific bacterial phyla was quantified using a real-time PCR (95°C 30 s; 55°C 30 s, 72°C 30 s; 55 cycles) and 16S rRNA gene-targeted phylum specific primers [43]. The total amount of *Eubacteria* [44] was used as an internal reference. The deep sequencing analysis was performed by LGC Genomics, Berlin, Germany using Illumina MiSeq V3 Chemistry (300 bp paired-end read) and Bacteria 16S (341F-785R) amplicon. The preparation of 16S rDNA templates, construction of Illumina libraries as well as the processing of reads occurred as described previously [21]. Combined reads were classified using "classifier.jar" from the Ribosomal Database Project [45] with a confidence cutoff of 50% and agglomerated at Genus level using RDPUtils [Quensen J. RDPutils: R Utilities for Processing RDPTool Output. R package version 1.2]. Frequencies of bacterial genera were estimated using the copy number-adjusted output and total bacterial counts. Heatmaps represent confidentially classified bacterial genera, with frequencies above 0.01% in non-cohoused BALB/c and below in non-cohoused ΔdblGATA-1 mice. The alpha and beta diversity estimation were performed without removing unclassified taxa and resampling of samples to equal sizes by the "vegan" (Oksanen, J. et al. Vegan: Community Ecology Package) and "phyloseq" [46] packages. The Shannon as well as the Simpson index were computed using the "estimate_richness" function. The non-metric multidimensional scaling was performed by the "ordinate" function in default parameter settings using the Bray-Curtis distance. Raw sequence data were deposited at the GEO database under the accession number PRJNA607006.

Preparation of microbiota filtrates

Fresh feces from BALB/c mice were collected and resuspended in ice-cold PBS. The fecal suspension was subsequently filtered through a 70 μm (Corning), 30 μm (Partec, Görlitz) cell strainer, 5 μm (Merck via Sigma, Darmstadt) and 0.45 μm (Sarstedt, Nümbrecht) bacterial syringe filters. After centrifugation, the pellet was added to the microbiota suspension of ΔdblGATA1 mice. Two hundred microliters of the final product was administered per mouse by gavage.

Co-cultivation of splenocytes with fecal filtrates

Total splenocytes were isolated from BALB/c mice, plated on 96-well plates (5×10^5 cells pro well) precoated with CD3 (3 μg/μL) and CD28 (3 μg/μL) antibodies and co-cultured with 0.45 μm filtrates from BALB/c or ΔdblGATA-1 mice. Forty-eight hours later the cells were reactivated with PMA (50 ng/mL) and ionomycin (5 μg/mL) for 2 h, washed, and resuspended in QIAzol Lysis Reagent for RNA extraction.

Fluorescent in situ hybridization

Intestinal mouse tissue biopsies were embedded in Tissue-Tek® O.C.T.™ Compound. Four micrometer thick sections were placed on SuperFrost plus slides, fixed with Carnoy's solution on ice for 20 min, and left to dry. Hybridization was performed in hybridization buffer (900 mM NaCl, 100 mM Tris-HCl (pH 8.0), 6.7 mM formamide, 346.7 mM SDS, 2 ng/μL DNA probe) at 50°C for 90 min. The slides were rinsed with de-ionized water, washed with washing buffer (900 mM NaCl, 100 mM Tris-HCl (pH 8.0), 346.7 mM SDS) for 5 min at 50°C, and rinsed again with de-ionized water. After drying, the sections were stained with DAPI solution (3.6 μM DAPI, 900 mM NaCl, 20 mM Tris-HCl) at room temperature for 5 min and rinsed with de-ionized water. Hybridization was performed in the a-Hyb hybridization station (Miltenyi Biotec). Dry sections were mounted and examined by fluorescence microscopy (Keyence Biorevo BZ-9000). The following probe conjugated with AlexaFluor 647 was used: AnaeroF1: 5'-ATGTAAAGTTCTTTTATCAG-3'.

Statistical analysis

Statistical analysis was done with GraphPad Prism 5.04. The corresponding tests are indicated in figure legends.

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Abbreviations: **PP**: Peyer's patches · **siLP**: small intestinal lamina propria · **Tfh**: T follicular helper

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